A null mutation in human CNTF gene is not causally related to neurological diseases
（ヒトにおける毛様体神経栄養因子の生理的及び病理的役割に 関する分子遺伝学的研究）

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主論文

A null mutation in human *CNTF* gene is not causally related to neurological diseases

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Summary

We report a null mutation in human ciliary neurotrophic factor gene (*CNTF*). In the mutated allele, transition from G to A in the intron produced a new splice acceptor site and an mRNA species coding for an aberrant protein. Analysis of tissue samples from various genotype subjects and transfection of *CNTF* minigenes into cultured cells demonstrated that the mutated allele expresses only the mutated mRNA species. Of the 391 Japanese people tested, 242 (61.9%) were normal homozygotes, 140 (35.8%) heterozygotes, and 9 (2.3%) mutant homozygotes; the mutated allele frequency was 20%. The frequency of mutant homozygotes and the distribution of the three genotypes were similar for the healthy and neurological disease subjects, indicating that human CNTF deficiency is not causally related to neurological diseases.

Ciliary neurotrophic factor (CNTF) originally was isolated as a target-derived survival factor of parasympathetic ciliary ganglion neurons[1-4]. Further studies have shown that it has a survival-promoting effect on a variety of neuronal cells, including sensory dorsal root ganglion neurons[2], pre- and postganglionic sympathetic neurons[2, 5], spinal motor neurons[6-10], hippocampal neurons[11], and medial septal neurons[12]. CNTF also induces cholinergic differentiation of sympathetic neurons *in vitro* [13] and promotes the differentiation of cultured bipotential O-2A progenitor cells to type-2 astrocytes[14, 15]. Although the richest sources of CNTF are myelin-producing Schwann cells in peripheral nerves[16-18] and ocular tissue[2, 19], astrocytes and other types of glias in the central nervous system produce detectable amounts of this factor[17, 20]. The α component of the CNTF receptor is widely expressed on the neurons of the central nervous system[21, 22]. Although a number of biological effects have been reported for CNTF, whether it is indispensable for the development and maintenance of nervous systems has yet to be shown.

A recent report showed that disruption of the *CNTF* gene in mice caused motor neuron degeneration[23], indicating an essential role of CNTF in the survival of motor neurons. Here we have identified a mutation in human CNTF gene (*CNTF*) that causes aberrant RNA splicing and abolishes the expression of CNTF protein. We have found four mutant homozygotes among 151 healthy subjects and five among 240 neurological disease subjects. The five homozygote patients suffered from essentially different diseases and had shown no apparent neurological abnormalities until their diseases manifested. In contrast to the report on mice, our findings indicate that CNTF deficiency is not causally related to any neurological disease in human.

Isolation of a mutated type of *CNTF* cDNA

A cDNA library was constructed from the poly(A)$^+$RNA of a sciatic nerve

obtained at autopsy. Screening of the library with a fragment of the *CNTF* gene showed two types of cDNA clones (Fig. 1). One contains an open reading frame for 200 amino acids with a calculated molecular mass of 23 kDa. The sequence of this clone was identical to the predicted exons of human genomic DNA[24-27], indicating that it is the cDNA for normal *CNTF*. The other type contains a 4 bp stretch insertion within the coding region, which causes a frameshift from the 39th amino acid with a stop codon 24 amino acids downstream. This mRNA therefore should code for an aberrant protein of 62 amino acids with a calculated molecular mass of 7 kDa. Both mRNAs had the same 38 N-terminal amino acids with different C-terminal peptides. As the inserted sequence (CCAG) is located at the splice acceptor site of an intron, the intron-exon boundary of the genomic DNA of the subject from whom the cDNA library was constructed was sequenced following PCR amplification. Two nucleotides, A and G, were detected at the -6 position of the intron (Fig. 2a), indicating that the nucleotide at this position is G for the normal allele but is replaced by A in the new *CNTF* allele. This mutation creates a new splice acceptor site and generates an mRNA species with the 4 bp insertion (Fig. 2b). Sequence analysis of the entire intron region revealed that the G to A transition was the only difference between the two alleles (data not shown).

Expression of *CNTF* mRNA from the normal and mutated alleles

Both the normal and mutated *CNTF* minigenes that carry the entire coding region and intron were isolated from genomic DNA by PCR amplification. The minigenes were ligated into a mammalian expression vector, pEF321[28], then introduced into Chinese hamster ovary (CHO) cells. *CNTF* mRNA species were analyzed using an RNase protection assay. ^{32}P-labeled RNA complementary to the mutated mRNA was prepared. As this probe completely hybridizes with the mutated mRNA, it would be protected from RNase digestion; whereas, when it hybridizes with the normal mRNA it would be

digested into two fragments (Fig. 3a). In CHO cells transfected with the normal minigene, the radioactive probe was digested into two fragments, indicating that all of the *CNTF* mRNA was the normal type. In CHO cells transfected with the mutated minigene none of the radioactive probe was digested, indicative that all of the mRNA was the mutated type (Fig. 3b).

We also did an RNase protection assay with human sciatic nerves obtained at autopsy. The genotypes of the subjects were determined by direct sequencing. The results show that all of the probe was digested into two fragments of the expected sizes in the normal homozygotes (N/N), but that in the heterozygotes (N/M) half of the probe was digested and half was not (Fig. 3b). This means that in the heterozygotes both the normal and mutated mRNAs were transcribed in nearly equivalent amounts.

After the above analysis had been completed, we were able to obtain tissue samples from a mutant homozygote subject who had died of amyotrophic lateral sclerosis (ALS) at the age of 62. Complementary DNAs for *CNTF* were amplified by RT-PCR using total RNAs extracted from the brain and muscle tissues as templates, after which they were differentially hybridized with probes specific to the normal and mutated cDNAs (Fig. 3c). In normal homozygote (N/N) only normal mRNA was detected, whereas both types of mRNA species were present in heterozygote (N/M). Only mutated mRNA was detected in the tissues from the mutant homozygote subject (M/M). These results show that the mutated allele produces only the mutated mRNA species.

Expression of CNTF protein from the normal and mutated allele

Antiserum against a CNTF fusion protein was prepared as described in *Methodology*. This antiserum recognized both the normal and mutated CNTF fusion proteins (Fig. 4a). Immunoblot analysis showed a single 23 kDa protein band in CHO cells transfected with the normal *CNTF* minigene, but there was no band in cells transfected with the mutated minigene (Fig. 4b). In the

extracts of sciatic nerves from the normal homozygotes (N/N) and heterozygotes (N/M), this antiserum detects two protein bands of 23 and 46 kDa that, respectively, presumably correspond to a monomer and dimer of normal CNTF[18, 27]. No band corresponding to mutated protein was detected (Fig. 4b). Immunohistochemical study was carried out with tissue samples from a normal homozygote and a mutant homozygote, and revealed that the Schwann cells in peripheral nerve tissue from the mutant homozygote subject completely lacked CNTF immunoreactivity, unlike those of the normal homozygote subject (Fig. 4c). These results show that the mutated allele produces neither normal nor mutated CNTF protein in a detectable amount.

Determination of *CNTF* genotypes in human subjects

This study has shown that there is a point mutation within the intron region which abolishes the expression of CNTF protein. It is important to determine whether this mutation is correlated with the incidence of neurological disease. Two procedures were developed for *CNTF* genotyping: One is allele-specific oligonucleotide hybridization (Fig. 5a). Alternatively, a genomic DNA fragment including the intron-exon boundary was amplified using two primers, the reverse primer being constructed to abolish an *Hae* III site in the second exon. This mutation eliminates an *Hae* III site in the intron. The PCR product of the normal allele therefore was digested by *Hae*III into two fragments, but the product of the mutated allele remained undigested (Fig. 5b).

We analyzed the *CNTF* genotypes of 391 human subjects; 151 healthy volunteers and 240 patients suffering from various neurological diseases, including 47, 30, and 52 respectively afflicted with ALS, Alzheimer disease, and Parkinson disease (Table). The frequency of the mutated allele was 20% in these Japanese subjects. Of the 151 healthy volunteers, 95 (62.9%) were normal homozygotes (N/N), 52 (34.4%) heterozygotes (N/M) and 4 (2.6%) mutant homozygotes (M/M). Healthy mutant homozygote subjects were 25, 30, 35 and

(Fig. 3c) There were five mutant homozygote subjects among the 240 patients with neurological diseases; two patients affected with ALS (their respective ages of disease onset being 60 and 71), one Parkinson disease (onset age of 63), one Guillain-Barré syndrome (onset age of 41), one peripheral neuropathy of unknown etiology (onset age of 15). None of these patients had shown neurological disturbances until their diseases manifested. Statistical analysis using *t* test revealed that the frequency of mutant homozygotes in ALS patients was not significantly higher than that in healthy volunteers ($P>0.05$).

Discussion

We have identified and characterized an RNA splicing mutation in the *CNTF* gene. A point mutation in the intron generated a new splice acceptor site and produced an mRNA species with a 4 bp stretch (CCAG) insertion within the region coding for CNTF. Although this mutated mRNA should code for 62 amino acids, no immunoreactive protein has been detected by immunoblot and immunohistochemical analyses of human peripheral nerves. These observations indicate that the mutated mRNA is efficiently transcribed, but the mutated protein is scarcely expressed. The mutated protein may be very unstable and rapidly degraded in the cells after translation.

Because the mutated allele conserves the normal splice acceptor sequence, it is possible that normal *CNTF* mRNA is transcribed along with the mutated mRNA species. Results of analyses of tissues from the three human genotypes and CHO cells transfected with the mutated *CNTF* minigene, however, negate this possibilitiy. Only the mutated mRNA species was detected in CHO cells transfected with the mutated *CNTF* minigene. In the sciatic nerves of the heterozygous subjects, both the normal and mutated *CNTF* mRNAs were equally expressed. RT-PCR and immunohistochemical analyses detected neither normal *CNTF* mRNA nor CNTF protein in tissues from a mutant homozygote subject. Although all the tissues of the mutant homozygote were

not examined, CNTF expression was completely suppressed in peripheral nerve, brain and muscle tissues, the main localization sites of CNTF[16-18, 20, 22]. Thus the mutation reported here is a null mutation and the mutant homozygote subjects are deficient in CNTF.

CNTF is thought to be particularly important for the survival of the motoneurons[6-10, 22]. Moreover, a recent report has shown that *CNTF* null mutant mice developed motor neuronopathy at 28 weeks of age[23]. In this study, however, we have found four mutant homozygotes in healthy subjects and five in the patients with various neurological diseases. The facts that these five patients suffered from essentially different neurological diseases and that the distribution of the three genotypes was similar for the healthy and neurological disease subjects indicate that the *CNTF* null mutation is not causally related to any neurological disease. Three mutant homozygote patients had claimed no neurological problems until 60, 63 and 71 of ages. In addition, we have found a healthy mutant homozygote subject of 50 years of age. This observation suggests that CNTF deficiency should not induce obvious neurologic dysfunction even at old age. Our findings in human seem contradictory to the report on CNTF deficient mice[23]. The loss of motoneurons in the mice, however, was rather small (22%), the muscle strength of the forelimbs being only slightly reduced. It could be that CNTF deficiency causes mild motor neuron degeneration and manifests obscure motor dysfunction. Detailed physiological tests may reveal slight functional disturbances in the CNTF deficient subjects.

There are other possible reasons for the apparent lack of neurological disturbance in homozygote subjects with the *CNTF* mutation. Other factors may compensate for the deficiency of CNTF. CNTF belongs to a family of hematopoietic cytokines that include leukemia inhibitory factor/cholinergic differentiation factor (LIF/CDF)[29]. The functional receptor complexes of CNTF

and LIF/CDF share common subunit molecules[22, 30, 31]. As both CNTF and LIF/CDF have similar effects on sympathetic neurons and motoneurons[9, 13, 32], LIF/CDF might compensate for a CNTF deficiency.

Moreover, if CNTF is dispensable under physiological conditions, human subjects who lack it can live without conspicuous symptoms. The predicted CNTF proteins of rat[3], rabbit[4], and humans[24-27] contain no signal peptides, which raises the question of how CNTF protein is released. Possibly it is released under pathological conditions such as nerve injuries in order to promote neuronal regeneration[3, 33]. Thus a CNTF deficiency may affect the manifestation, course, and prognosis of neurological diseases.

We have, for the first time, detected a *CNTF* null mutation and identified nine mutant homozygote subjects. Results of *CNTF* genotyping of various types of subjects indicate that this mutation is not causally related to any neurological disease. The following are now being done: (i) *CNTF* genotyping of more subjects to identify mutant homozygotes among healthy persons as well as among patients having various diseases; (ii) detailed physiological testing of mutant homozygotes to detect slight functional disturbances; (iii) histopathological analysis of postmortem specimens from mutant homozygotes; (iv) a search for additional null mutations in the *CNTF* gene. The results of these studies, together with the observations reported here, should provide important information about the physiological and pathological roles of CNTF in the human nervous system.

Methodology

Subjects. Human tissue samples were obtained at autopsy. Blood samples were taken mainly from patients admitted to the Tokyo Metropolitan Neurological Hospital. The diagnoses of the patients' neurological diseases were based on clinical evidence. Patients with PROBABLE Alzheimer disease according to the NINCDS-ADRDA criteria[34]were studied. Genomic DNA was extracted from sciatic nerve and liver specimens, and from leukocytes of healthy volunteers as described elsewhere[35]. All the subjects were of Japanese ancestry.

Construction and screening of a cDNA library. Total RNA was extracted from human sciatic nerves with guanidinium thiocyanate and purified by centrifugation in a 5.7 M CsCl solution. Poly(A)$^+$RNA was selected by oligo(dT) cellulose column chromatography. An oligo(dT) primed cDNA library was constructed with a cDNA synthesis kit. The 379 bp fragment of the *CNTF* coding region was prepared by PCR with human genomic DNA as the template then ^{32}P-labeled with a multiprime labeling kit and used as the probe. Screening of 8 x 10^5 independent recombinant phages gave 23 positive clones. Four clones that had an insert longer than 0.7 kbp were subcloned into pUC18.

Analyses of cDNA, genomic DNA clones, and PCR products. The cloned cDNAs were sequenced on both strands with a deaza T7 sequencing kit (Pharmacia). For direct sequencing, a 224 bp fragment that included the intron-exon boundary was amplified by asymmetric PCR. The forward primer (5'-CCTTGGCCAGTGAGATGAG-3') was selected within the intron[25]. The reverse primer (5'-CTTGAAGGTTCTCTTGGAGT-3') corresponded to the sequence in the second exon. A DNA thermal cycler (Perkin-Elmer, Cetus) was

used to run thirty cycles of the reaction (94°C for 40 sec, 55°C for 2 min and 72°C for 3 min) in a 50µl reaction mixture containing 0.5 µg of genomic DNA and 50 pmoles of both primers. The product was purified on a 3% Nusieve GTG agarose gel. Asymmetric PCR was performed as above with the purified product as the template together with 0.5 pmole of the forward primer and 50 pmoles of the reverse primer. The resulting single strand DNA was sequenced using the primer within the product.

RNase protection assay. The RNase protection assay was done as described elsewhere[36]. A 290 bp fragment of the mutated CNTF cDNA containing the 48 bp of the 5'-untranslated region was subcloned into the *EcoR*I and *Hind*III sites of pSPT19 (Boehringer). The vector was linearized with *EcoR*I, and the RNA probe was synthesized using [α-^{32}P] CTP (60 µM, 200 Ci/mmol) and T7 RNA polymerase. Total RNA (20 µg from sciatic nerves and 2 µg from CHO cells) was hybridized with 5 x 10^5 cpm of the radioactive RNA probe overnight at 45°C, after which it was digested with RNase A (40 µg/ml) and RNase T1 (360 units/ml) at 37°C for 30 min. The reaction products were separated on a 6% polyacrylamide-7 M urea gel, then dried and subjected to autoradiography at -80°C.

Immunochemical procedures. The coding region of the normal *CNTF* cDNA was amplified by PCR using a forward primer (5'-ACCCCCGGGAGCTTTCACAGAGCATTCACC-3') and a reverse primer (5'-ACGGAATTCTTACATTTTCTTGTTGTTAGC-3'). The PCR product was digested with *Sma*I and *EoR*I, then unidirectionally ligated into the prokaryotic expression vector pGEX-2T[37]. Upon induction with IPTG (0.2 µg/ml) for 2 h, E. coli DH5α expressed a large amount of a fusion protein composed of glutathione

S-transferase (GST) and CNTF protein. This protein was purified as described elsewhere[37]. By immunizing mice with the fusion protein, we obtained CNTF-specific antiserum. The antiserum was preabsorbed with GST protein. Extracts of sciatic nerves and CHO cells containing 50 µg protein were separated electrophoretically on a 5-20% polyacrylamide gradient gel under reduction conditions. Immunodetection was done with an ECL Western blotting system (Amersham). The immunohistochemistry of 8µm fresh frozen sections of nerve root tissue was determined with a Vectastain ABC kit (Vector).

RT-PCR followed by differential oligonucleotide hybridization. Total RNAs were extracted from brain (occipital lobes of the cerebrum) and muscle (m. iliopsoas) tissues obtained at autopsy. First-strand cDNAs were prepared with AMV reverse transcriptase (Life Science). PCR was done with a set of primers corresponding to the first and second exons of *CNTF*. The RT-PCR products were vacuoblotted onto a nylon membrane and hybridized to specific oligonucleotides[38]; 5'-CCCTGATGCTTCACATAGGA-3' for the normal cDNA and 5'-GCTTCACCTGGATAGGAT-3' for the mutated cDNA.

Isolation and expression of *CNTF* minigenes. The gene fragment carrying two exons and an intron was amplified with a forward primer (5'-CGGAATTCATATGGCTTTCACAGAGCATTC-3') and with the reverse primer used for procaryotic expression. Genomic DNA from a heterozygote subject was used as the template. After digestion with *EcoR*I, the PCR product was ligated into the *EcoR*I site of BluescriptSK(-). DNA clones having the normal and mutated alleles were detected by differential hybridization with specific oligonucleotides[38]; 5'-TCACCTGGCCGAGGATACA-3' for the normal allele and 5'-TCACCTGGCTGAGGATACA-3' for the mutated *CNTF* allele. The normal and mutated minigenes were confirmed by sequencing, then ligated

into a mammalian expression vector, pEF321, and expressed in CHO cells.

Determination of genotypes. For differential hybridization, the genomic DNA that included the mutation was amplified, and the PCR products were hybridized with the oligonucleotide probes specific to the normal and mutated alleles as described above. For the restriction analysis of the genotypes, genomic DNA was amplified by PCR using the forward primer from 1067 to 1090 in the intron (Fig. 2) and the reverse primer, 5'-CAGGTTGATGTTCTTGTTCATGCCC-3'. This reverse primer was complementary to the second exon (126-150 nt in Fig. 1), in which thymidine (underlined) was substituted for G to abolish an intrinsic *Hae*III site. The PCR products were digested with *Hae*III then separated electrophoretically on a 3% agarose gel.

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Legends for Figures

Fig. 1 The nucleotide and predicted amino acid sequences of the normal and mutated cDNAs for *CNTF*. Amino acid sequences of the normal CNTF shown in the uppermost lines in single-letter notation are numbered with open arrowheads. The nucleotide sequences of normal *CNTF* cDNA are shown in the second line. The four base insertion in the mutated cDNA is boxed. Amino acid sequences of the mutated CNTF (lower lines) are numbered with closed arrowheads. The mutated *CNTF* cDNA specific sequence generated after the insertion is shown in bold type. Asterisks indicate stop codons.

Fig. 2 Analysis of the intron-exon boundary of the *CNTF* gene. a. Direct sequence analysis of the *CNTF* gene of a subject from whom both normal and mutated cDNAs had been isolated. The point mutation (G to A) is boxed. b. The new splice acceptor site of the mutated allele, as compared to the normal splice acceptor site. Uppercase and lowercase letters respectively correspond to the exonic and intronic sequences. The substituted adenine is shown in bold type and underlined.

Fig. 3. RNase protection and RT-PCR analyses of the *CNTF* mRNAs from the normal and mutated alleles. a. Schematic representation of the cRNA probe for RNase protection and the normal and mutated *CNTF* mRNAs. Dashed, open, and closed areas respectively indicate the 5'-untranslated region, the coding region, and the inserted 4 bp sequence of the mutated mRNA. The size of the probe fragments protected by RNA from human sciatic nerves and*CNTF* minigene transfectants is shown. Note that the minigenes do not contain the 48bp of 5'-untranslated region. b. RNase protection analysis of RNAs from the CHO cells transfected with *CNTF* minigenes and human sciatic nerves.

Molecular size marker was ^{32}P-labeled ϕX174/Hinf I digest. N indicates the normal minigene; M, the mutated minigene; N/N, the normal homozygote; N/M, the heterozygote. The fragments of the probe protected by the normal and mutated *CNTF* mRNA are respectively indicated by closed and open arrowheads. The probe was not protected by either the RNA extracted from mock-transfected CHO cells or tRNA (data not shown). c. RT-PCR analysis of tissue samples (b; brain, m; muscle) obtained from a normal homozygote (N/N), a heterozygote (N/M), and a mutant homozygote (M/M) subject. *CNTF* mRNAs were amplified by RT-PCR, then differentially hybridized to the specific probes, and subjected to autoradiography. Cloned mutated and normal *CNTF* cDNAs were the positive controls.

Fig. 4 a. Immunoblot analysis of CNTF-GST fusion proteins by a CNTF-specific antiserum. Coomasie blue stained gel (panel 1) and immunoblot (panel 2) are shown. G indicates glutathione S-transferase (GST) protein, N, normal CNTF-GST fusion protein, M, mutated CNTF-GST fusion protein. Because mutated CNTF fusion protein was insoluble, bacterial lysate was used instead of a purified protein. The antiserum preabsorbed with GST recognizes normal (closed arrowhead) and mutated (open arrowhead) CNTF-GST fusion proteins, but not GST (panel 2). The low molecular weight bands in lane M (panel 2) are presumably degraded products of mutated CNTF protein, because they are also detected by the antiserum raised against a synthetic peptide corresponding to the residues 39-62 of mutated CNTF protein (data not shown). b. Immunoblot analysis of the extracts from CHO cells transfected with minigenes and human sciatic nerves. N indicates the normal minigene; M, the mutant minigene; N/N, the normal homozygote; N/M, the heterozygote. The 23 kDa and 46 kDa protein bands, respectively, presumably correspond to the monomer and dimer of normal CNTF (arrows). c. Immunohistochemical demonstration of CNTF

in spinal cord anterior roots (L3 level). Specimens were obtained 10-11 hours postmortem from a normal homozygote (N/N) and a mutant homozygote (M/M) subject who died of ALS at the respective ages of 70 and 62. In the normal homozygote (N/N), CNTF antiserum has specifically stained the Schwann cells surrounding the myelin sheaths in both the longitudinal and transverse sections. In contrast, no CNTF immnoreactivity is present in the Schwann cells from the mutant homozygote (M/M). Vascular endothelial cells and erythrocytes are strongly stained because of their intrinsic peroxidase activity (M/M specimens stained by CNTF antiserum). Immunoreactivity is shown in the Schwann cells of both the normal and mutant homozygotes by the polyclonal antibody specific to S100 protein (Dako). All micrographs at magnification x600. Essentially the same results were obtained with spinal cord posterior root samples (data not shown).

Fig. 5 Determination of the genotypes for *CNTF*. N/N the normal homozygote, N/M the heterozygote, and M/M the mutant homozygote. a. Allele-specific oligonucleotide hybridization method. b. Restriction digestion analysis using *Hae*III. The PCR product (134 bp) derived from the normal allele is digested by *Hae*III into two fragments of 94 bp and 40 bp, but the PCR product of the mutated allele is not digested.

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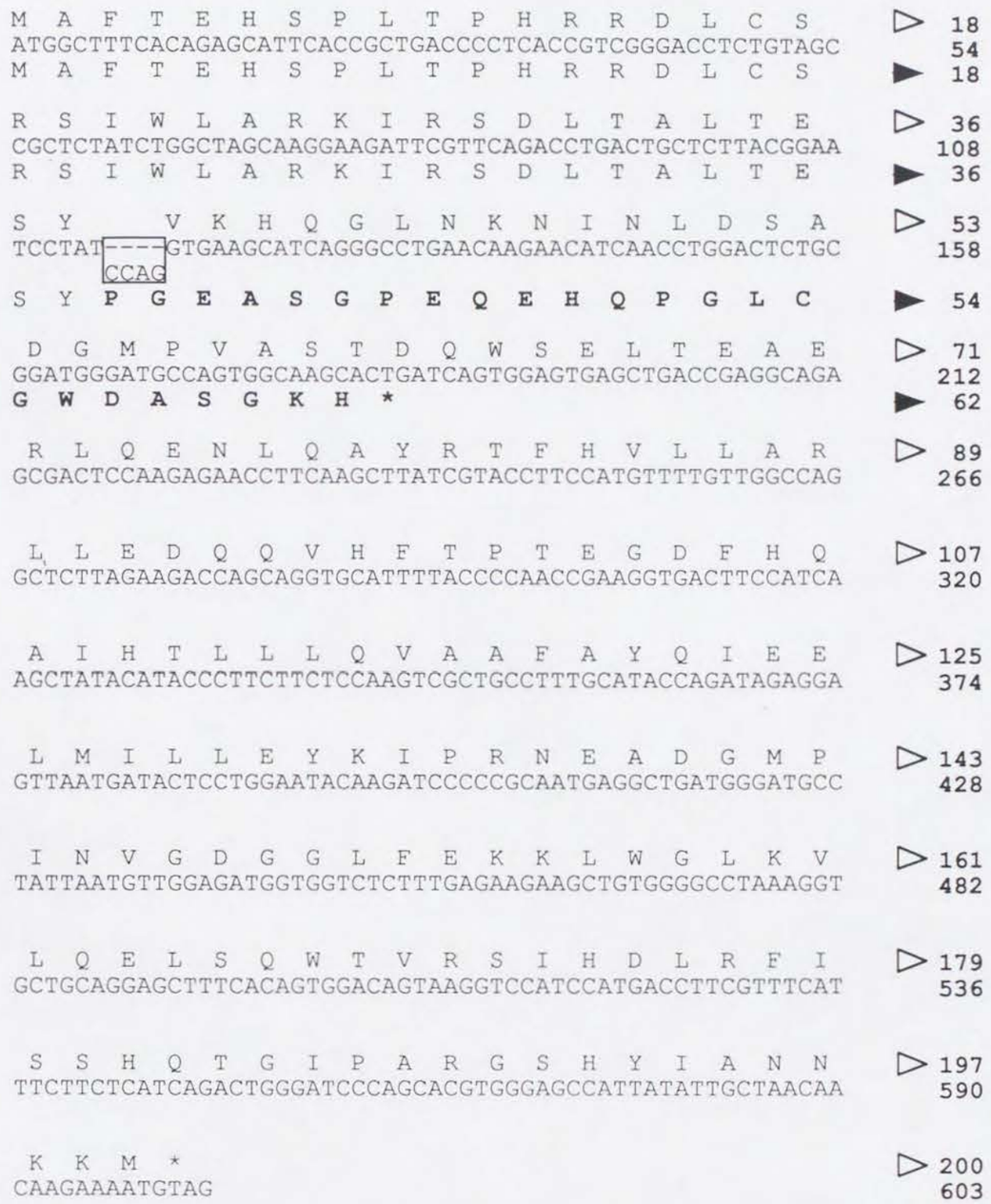
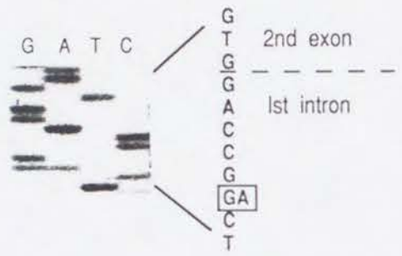


Fig. 1
(TAKAHASHI et al)

a



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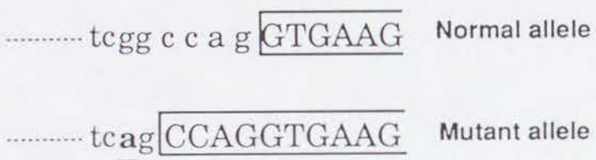
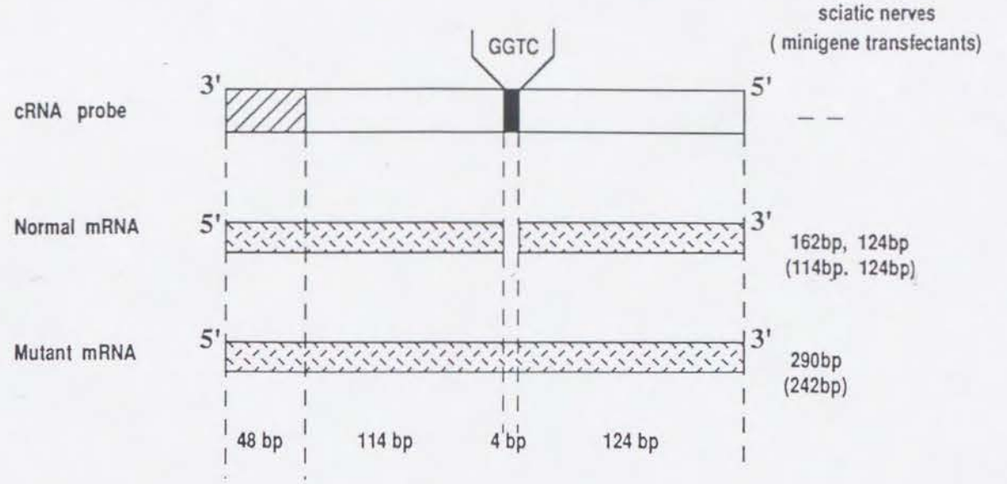


Fig. 2
(TAKAHASHI et al)

a



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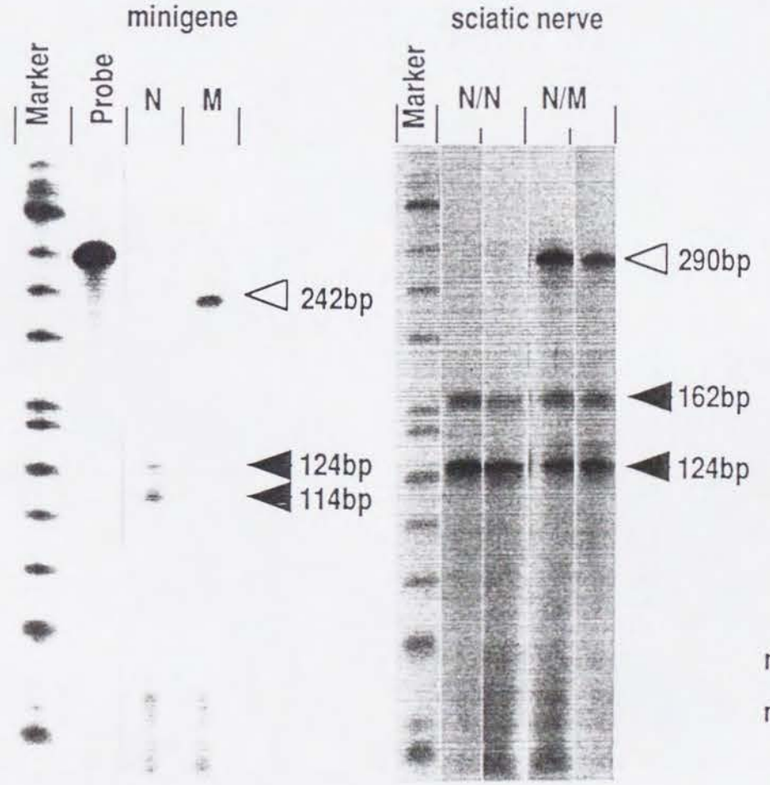


c

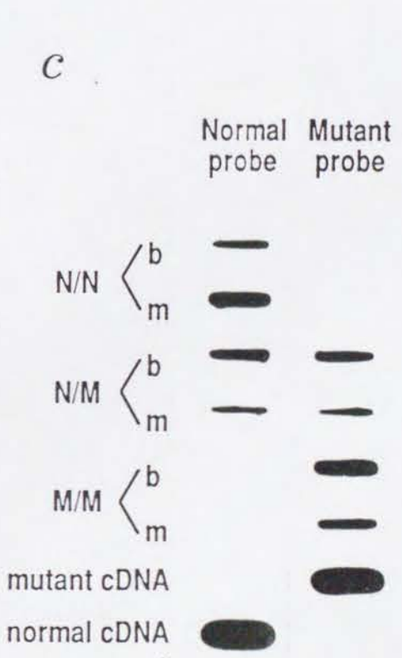
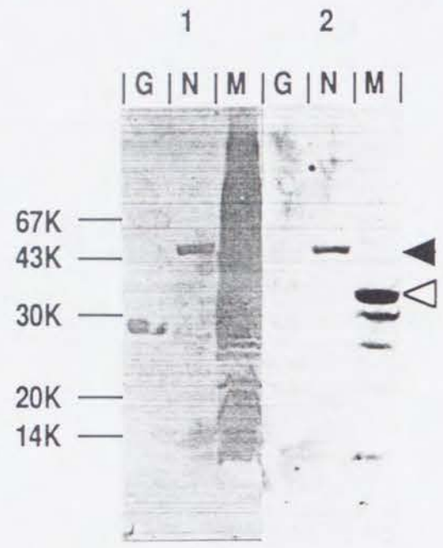


Fig.3 (TAKAHASHI)

a



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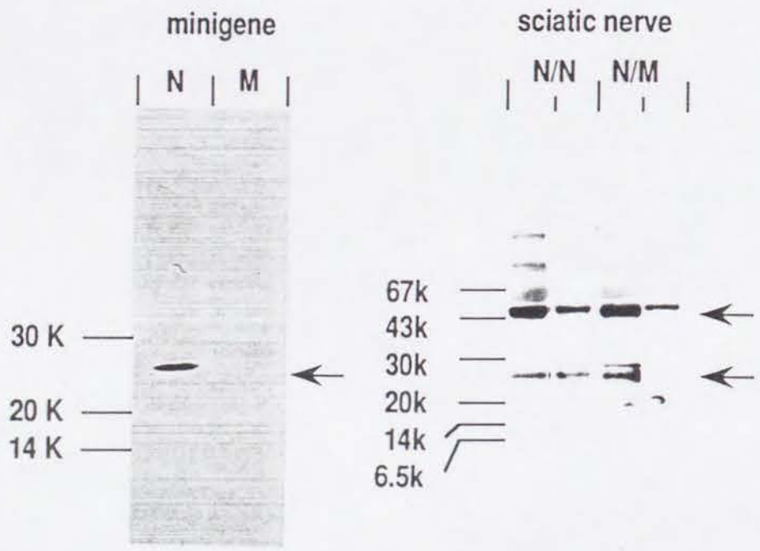

Fig. 4
(TAKAHASHI)

Staining ε CNTF antibody

c

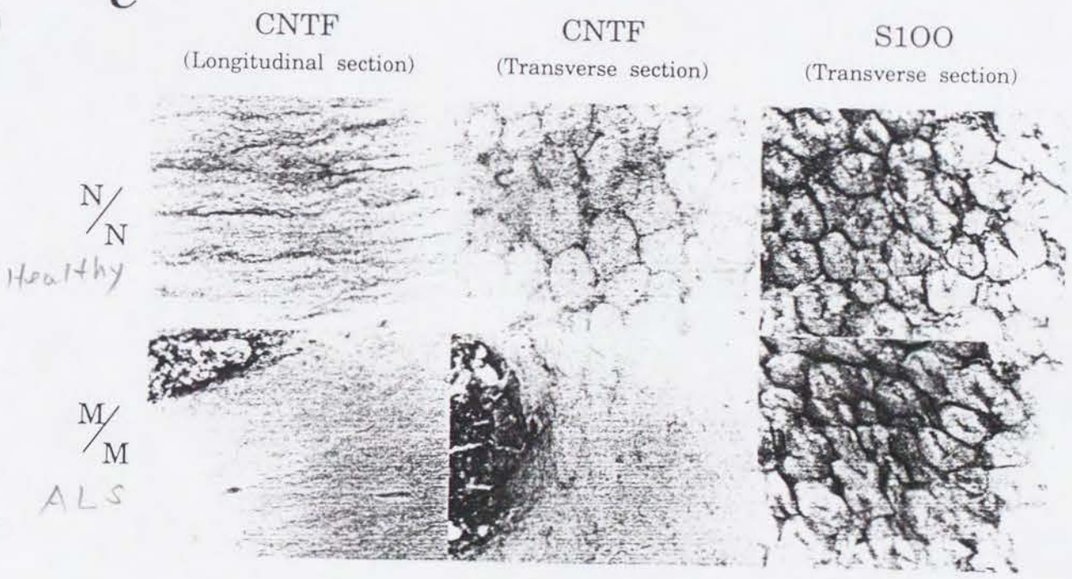

Fig. 4c
(TAKAHASHI et al)

Table : Distribution of CNTF genotypes in healthy subjects and patients with neurological diseases

Subjects \ Genotype	Number of subjects (%)		
	N/N	N/M	M/M
Healthy volunteer	95 (62.9)	52 (34.4)	4 (2.6)
ALS	27 (57.4)	18 (38.3)	2 (4.3)
Alzheimer disease	17 (56.7)	13 (43.3)	0 (0)
Parkinson disease	30 (57.7)	21 (40.4)	1 (1.9)
Miscellaneous diseases	73 (65.8)	36 (32.4)	2 (1.8)
Total	242 (61.9)	140 (35.8)	9 (2.3)

Fig. 5
(TAKAHASHI et al)